SPECIFICATION

SYNTHESIS OF CORE SUGAR CHAIN STRUCTURE OF ASPARAGINE-LINKED GLYCOPROTEIN

TECHNICAL FIELD

The present invention relates to a field of chemical synthesis of sugar chain, and specifically, to a convenient method for chemically synthesizing sugar chains of glycoprotein and a synthetic intermediate thereof.

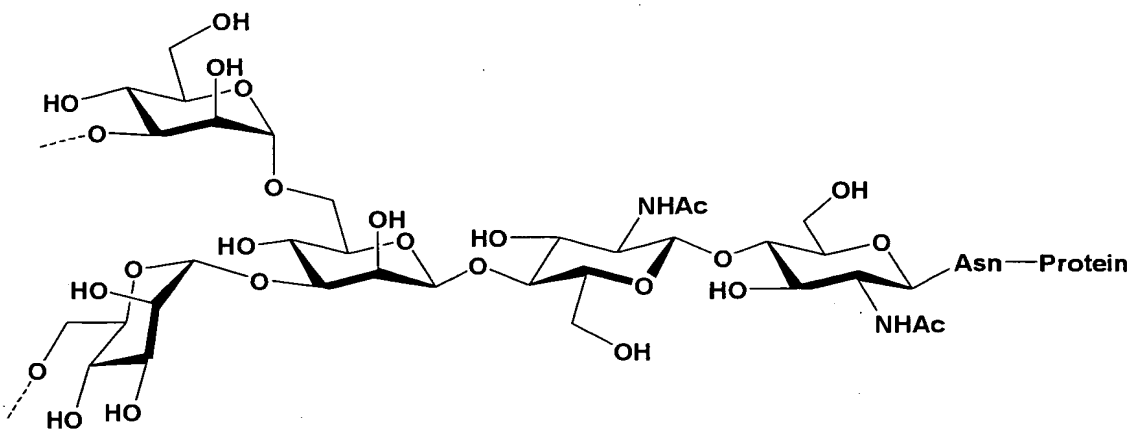
BACKGROUND ART

Glycoprotein means a protein comprising a moiety of oligosaccharide referred to as a sugar chain.

Recently, glycoprotein has been found to be closely involved with biological processes such as cell adhesion or signaling, and structures of sugar chains which trigger various biological processes have gradually emerged. However, only a small amount of glycoprotein is expressed in a living body for the sugar chain to mediate a biological process, and it is quite difficult to obtain pure glycoprotein in sufficient quantity to determine the chemical and physical properties of the sugar chain.

An asparagine-linked glycoprotein is one of the glycoproteins and ubiquitously found in human serum or ovalbumin. The asparagine-linked glycoprotein is classified into a high mannose-type, a complex type and a mixed type according to characteristics of a branch of the sugar chain and/or constituting sugar. All of these types have a common core sugar chain structure of a penta-saccharide comprising

three molecules of mannose and two molecules of N-acetyl glycosamine at the reducing terminal of the chain;



the core sugar chain structure of asparagine-linked glycoprotein

Accordingly, chemical synthesis of the core sugar chain structure shown in the formula above provides the basis for studying the function of asparagine-linked sugar chain.

DISCLOSURE OF INVENTION

However, an efficient method for synthesizing the core sugar chain structure of the asparagine-linked sugar chain is not yet known. One of the reasons is that the core sugar chain structure contains a moiety chemical synthesis of which is quite difficult.

In the chemical synthesis of the core sugar chain structure, it is extremely difficult to form a bond of mannose β-glycoside, that is a bond of β-manno-glycoside (Manβ1→4-GlcNAc). The reason comes from the facts that a neighboring group effect is not available since 2-OH group of mannose is linked at the axial position and the β-manno glycoside bond brought an electrically unstable structure against an anomer effect typically found in sugars. Kunz et al. discloses a chemical method for preparing a β-manno glycoside structure, which

contains a complicated process and requires the time and cost of running (Kunz, H. and Gunther, W. (1988) Angew. Chem. Int. Ed. Engl. 27, 1086-1087).

Other reasons why a bond of β-manno glycoside (Manβ1→4-GlcNAc) is difficult to be formed are that the acceptor of the glycosilation reaction is N-acetyl glucosamine of low solubility in the reaction medium and the reactivity of 4-OH group is low compared with the other OH groups (reactivities of OH groups; 1-OH>>6-OH>>2-OH>3-OH>4-OH).

In addition, synthesis of a structure of GlcNAc β 1→4GlcNAc has some problems in the chemical synthesis of the core sugar chain structure of asparagine-linked sugar chain.

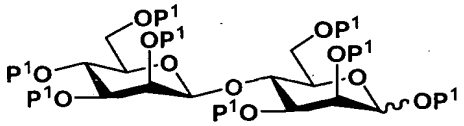
As described above, synthesis of the trisaccharide (Man β 1→4GlcNAc β 1→4GlcNAc) at the reducing terminal especially remains as a big problem in the chemical synthesis of the core sugar chain structure of asparagine-linked sugar chain. In order to synthesize the core sugar chain structure, how to synthesize the β-manno glycoside bond effectively is especially an issue to be solved.

The inventors of the present invention focused their attention to a natural polysaccharide having the structure of mannoside β 1→4 bonds, especially galactomannan, guar gum and mannan, which have mannoside β 1→4 bonds.

The objective of the present invention is to break the primary barrier in the synthesis of the core sugar chain structure, specifically to form a β-manno-glycoside bond by using a disaccharide unit of Man β 1 →4Man comprised in a structure of natural polysaccharides, and to establish an efficient method for synthesizing the structure of the core sugar chain.

As a result, the present invention relates to a method comprising,

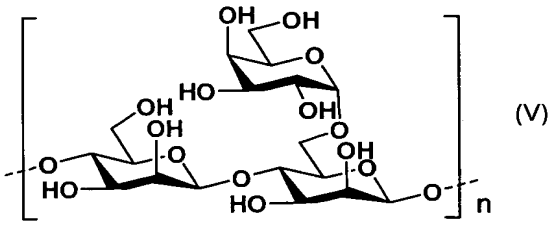
(1) a process of preparing a compound of mannose disaccharide(a type of ManP^{1}β1→4ManP^1)shown with a formula (I);



(I)

wherein P^1 is an OH-protecting group and the wavy line means that -OP^1 is linked at an axial or equatorial position or mixture of both,

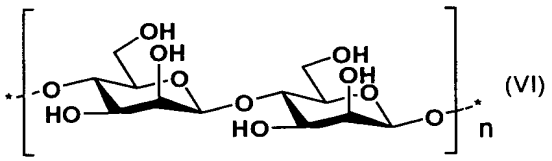
by hydrolyzing a polysaccharide having mannose β-1,4-bonds, preferably galactomannan, guar gum or mannan having mannose β-1,4-bonds, more preferably a galactomannan derivative shown with a formula (V);



(V)

(n is an integer of 50 or more)

or a mannan derivative shown with a formula (VI);



(VI)

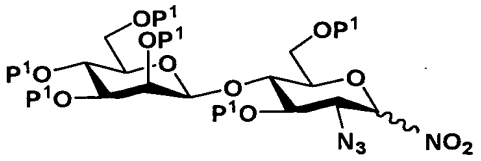
(n is an integer of 50 or more)

and protecting hydroxyl groups of the resulted compound,

(2) each process for converting the obtained mannose disaccharide compound (a type of ManP^{1}β1→4ManP^1) into a glycal compound in which mannose at the reducing terminal of the mannose disaccharide compound is changed into a glycal, by halogenating and reducing the mannose disaccharide, and

(3) preparing an azide disaccharide compound (a type of ManP^{1}β1

→4ManP[1]) shown with a formula (II) in which the 2-azide group of mannose in the reducing terminal is linked at the equatorial position;



(II)

wherein P[1] is the same above, and the wavy line means that $-NO_2$ is linked at an axial or equatorial position or mixture of both,

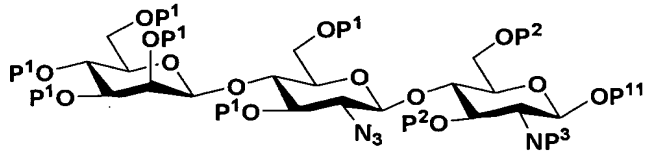
by azidenitration reaction of the glycal compound above,

(4) each process for substituting the nitro group of the azide disaccharide compound (a type of ManP[1]β1→4ManP[1]) with a leaving group, preferably

(4-1) substituting the nitro group of the azide disaccharide compound (a type of ManP[1]β1→4ManP[1]) with a $-OP^{10}$ group (P^{10} is an OH-protecting group), and preparing a trihaloacetoimidate derivative by reacting with trihaloacetonitrile after removal of the P^{10} group, or

(4-2) substituting the nitro group of the azide disaccharide compound (a type of ManP[1]β1→4ManP[1]) with a leaving group, and

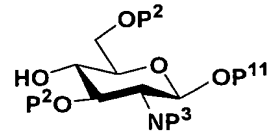
(5) preparing a trisaccharide compound (a type of Manβ1→4GlcNP[1]β1→4GlcNP[2]) shown with a formula (III);



(III)

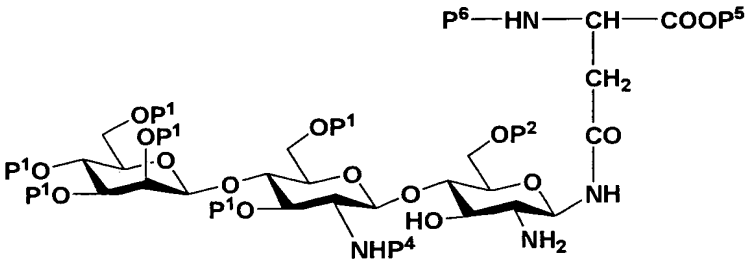
wherein P[1], P[2], P[3] and P[11] are the same above,

by reacting the derivative above in which a leaving group was introduced with amino-protected glucopyranoside of the formula



wherein P^2 and P^{11} are an OH-protecting group and P^3 is an amino-protecting group, and

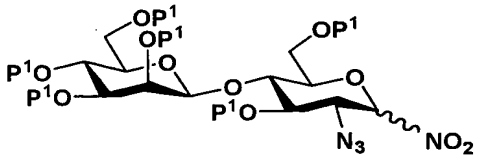
(6) a process for preparing an asparagine-linked trisaccharide compound (Manβ1→4GlcNP$^1\beta$1→4GlcNP^2) shown with a formula (IV);



(IV)

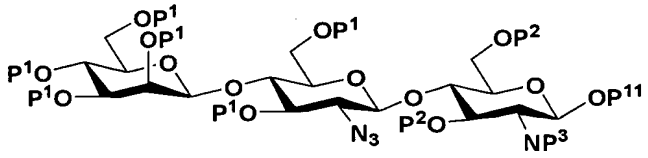
wherein P^1 and P^2 are the same above, P^4 and P^6 are independently amino protecting groups and P^5 is a carboxyl-protecting group, and a method of each process, when preparing the trisaccharide (Man β 1→4GlcN β 1→4GlcN) at the reducing terminal of the core sugar chain structure in the asparagine-linked glycoprotein.

Furthermore, the present invention relates to the azide disaccharide compound (a type of ManP$^1\beta$1→4ManP^1) of the formula (II) which is a useful synthetic intermediate in the methods of the present invention;



(II)

wherein P^1 is an OH-protecting group, the wavy line means that $-NO_2$ is linked at an axial or equatorial position or mixture of both, and to the trisaccharide compound shown with the formula (III);



(III)

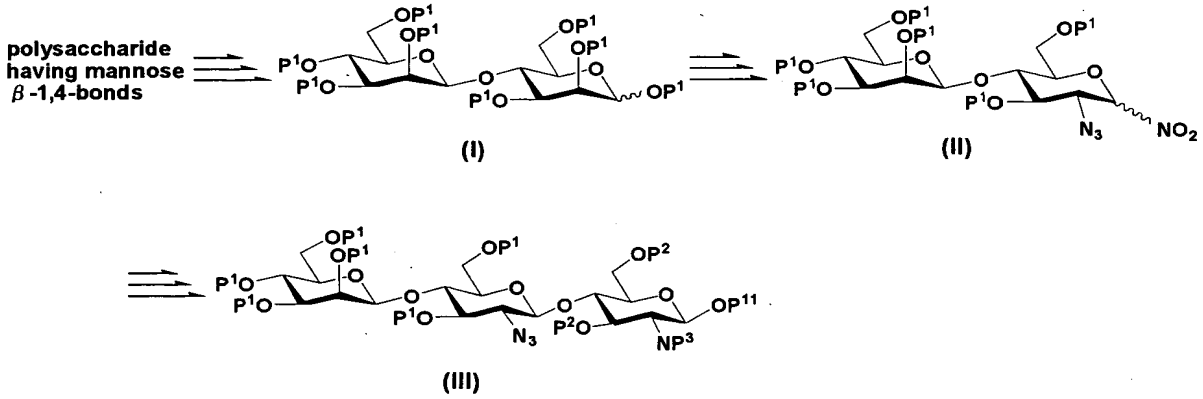
wherein P^1, P^2 and P^{11} are OH-protecting groups and P^3 is an amino-

protecting group.

According to the present invention, the trisaccharide moiety(Manβ1→4GlcNβ1→4GlcN) of the reducing terminal in the core sugar chain structure of the asparagine-linked glycoprotein sugar chain is easily synthesized and it is useful to clarify the function and structure-characteristics of the asparagine-linked glycoprotein which causes various life processes.

BEST MODE FOR CARRYING OUT THE INVENTION

The outline of the novel route for synthesizing the core sugar chain structure in the present invention is depicted as follows;



wherein the wavy line means that the -OP^1 or nitro group is linked at an axial or equatorial position or mixture of both.

At first, the disaccharide compound (I), Manβ1→4Man, is obtained after acid hydrolysis of a polysaccharide having mannose β-1,4-bonds and acetylation of the product. Next, it is converted to the glycal derivative wherein mannose of the reducing terminal was converted to glycal by a chemical method, and followed by azide nitration reaction to give the compound (II). The compound (II) which has the equatorial 2-azide group at the reducing terminal can be transformed to the moiety of Manβ1→4GlcNAc in the core sugar chain

structure and is a useful key intermediate.

Thus, the intermediate (II) is easily converted to the moiety of Manβ1→4GlcNAc which is difficult to prepare through other synthetic scheme, while the intermediate (II) can be easily prepared in a large scale at a reasonable cost from the compound (I), which is available from galactomannan, guar gum or mannan derivatives. Furthermore, the trisaccharide compound (III), which is ready to be converted to the trisaccharide (Manβ1→4GlcNAcβ1→4GlcNAc) of the reducing terminal in the core sugar chain structure, is synthesized when the intermediate (II) is used as a glycosyl donor.

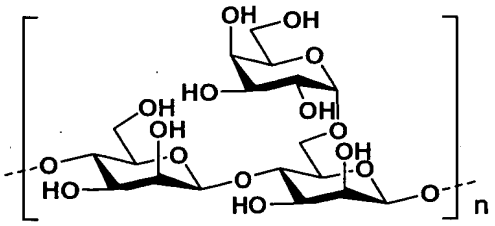
Thus, the inventors of the present invention succeeded to simplify the scheme for synthesizing the trisaccharide of the reducing terminal in the core structure by utilizing a natural polysaccharide available at a low cost.

In the following description, each process of the present invention is explained in detail.

Process (1)

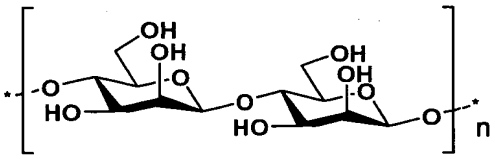
In Process (1), the compound (I) of mannose disaccharide ($ManP^1\beta1\rightarrow4ManP^1$) is prepared from a polysaccharide having mannoseβ-1,4-bonds. At first, a polysaccharide having mannoseβ-1,4-bonds is hydrolyzed, the OH groups are protected and the desired disaccharide is isolated.

As a starting material, a polysaccharide having mannoseβ-1,4-bonds, preferably galactomannan, guar gum or mannan having mannoseβ-1,4-bonds, more preferably a galactomannan derivative of the formula (V);


(V)

whrein n is an integer of 50 or more

or a mannan derivative of the formula (VI);


(VI)

wherein n is an integer of 50 or more

is used.

Galactomannan derivatives (also referred to as galactomannoglycan) are extensively present in seeds of legume family, e.g., alfalfa or clover. Galactomannan in the seeds of guar (Cyamopsis tetragonolobus) and carob or locust bean (Ceratonia siliqua) is available in the market as gum products derived from plants.

Guar gum extracted from guar seeds is a natural polysaccharide having a straight sugar chain comprising a series of mannose β 1→4 bonds wherein galactose is linked through α 1→6bond to every mannose residue as a branch. Almost uses of this material are food additives such as thickeners of various canned products, quality improving agent (inhibitor of shape-loosing) or taste-regulator of various foods and easily available at an extremely low cost.

A mannan derivative is a generic name of polysaccharides comprised of D-mannose. Plant mannan derivatives contained in endosperm of ivory nut or bulbs of orchidaceous plant have straight chain structure in which D-mannose residues are linked through β 1→ 4 bonds and insoluble in water.

In detail, these are described in "Comprehensive Dictionary for Utilization of Regional Biological Resources", Ed., Hiroshi Fujimaki, 1998, Rural Culture Association; Y. C. Lee, et al. (1977) Analytical Biochem., 79, 329-337; and Shiryo Yaga, et al. (1995) Mokuzai Gakkaishi, vol.41, No 4, 440-443.

Usually, acid hydrolysis is applied to hydrolyze polysaccharides having mannoseβ-1,4-bonds. For the purpose, sulfuric acid, preferably 10-20% sulfuric acid, trifluoroacetic acid or sulfuric acid-acetic acid is used and the reaction temperature of 50-70°C is preferable.

Materials of which the polymerization degree is equal or more than 9 are removed by isolation of galactomannan soluble in 70%EtOH. In general, the polymerization revel is increased the more, the derivative remains in the insoluble residue.

In order to protect the hydroxyl group, acetyl, benzyl, 4-methoxybenzyl, benzoyl, methoxymethyl, tetrahydropiranyl, torimethylsilyl, and triethylsilyl group etc., are usually used.

Isolation of the disaccharide is achieved by silica gel chromatography and/or HPLC.

Process 2

In Process 2, a glycal compound is prepared from the mannose disaccharide compound (I) (a type of $ManP^1\beta1\rightarrow4ManP^1$). At first, the glycal compound is prepared from the disaccharide by halogenation and successive reduction of the position 1 in mannose of the reducing terminal. Usually, mannose is halogenated around r.t. using hydrogen halide or acid halide etc. Reduction is carried out by using a metal such as zinc etc., while avoiding a reaction at high temperature.

Process 3

In Process 3, the azide disaccharide compound (II), in which the

2-azide group of mannose in the reducing terminal is linked at the equatorial position, is prepared by azidenitration reaction of the glycal compound.

The azidenitration reaction is carried out by simultaneous azidation and nitration. A mixture of equatorial and axial isomers is provided, and the compound having the 2-azide group of mannose in the reducing terminal at the equatorial position is isolated by purifying the mixture.

Process 4

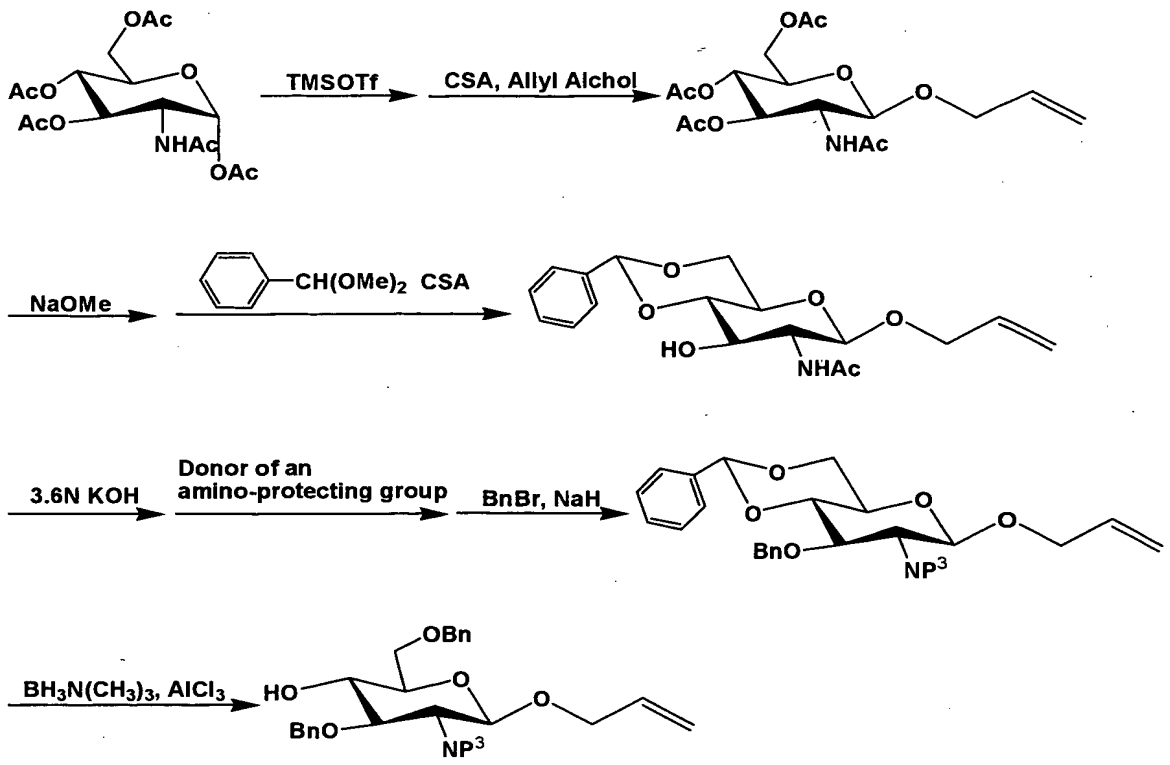
In Process 4 the nitro group in the azide disaccharide compound is substituted for a leaving group, which generally includes fluorine, bromine, chlorine, trichloroacetoimidate, 4-pentenyl, alkylthio (sulfur) and arylthio.

Preferably, the nitro group in the azide disaccharide compound is substituted for the $-OP^{10}$ group (P^{10} is an OH-protecting group), and the trihaloimidate derivative is obtained by the reaction with trihaloacetonitrile after removal of the P^{10} group or a halogenated derivative is obtained by the reaction with hydrogen halide. Alternatively, the $-OP^{10}$ derivative or P^{10}-deprotected derivative may be converted to the derivatives having a leaving group such as a penteny, acetylthio or arylthio group.

Process 5

In Process 5, the resulting derivative having a leaving group is reacted with amino-protected glucopyranoside to prepare the trisaccharide compound (a type of $Man\beta1\rightarrow4GlcNP^1\beta1\rightarrow4GlcNP^2$).

The amino-protected glucopyranoside may be prepared according to the following scheme.

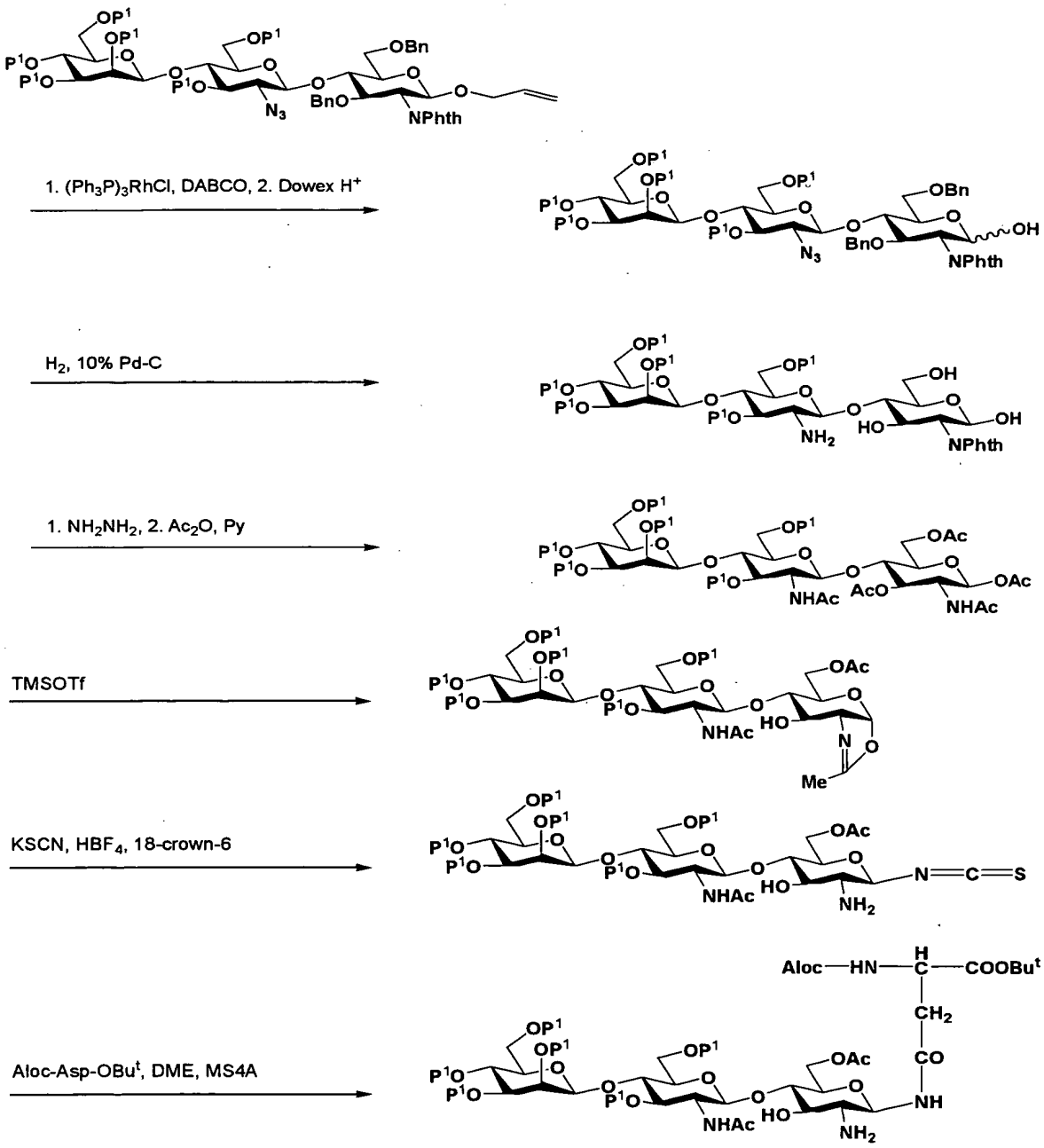


As the P^3 group which is an amino protecting group, phthalimide, tert-butyloxycarbonyl, benzyloxycarbonyl, acetyl, benzoyl or benzyl group etc. is usually used.

Next, this compound is reacted with the above derivative having a leaving group under acidic (Lewis-acidic) condition.

Process 6

In Process 6, the trisaccharide compound is coupled with asparagine. The coupling with asparagines is carried out according to the next scheme, for example.



The trisaccharide prepared above may be coupled with an asparagine residue of the desired protein and the sugar chain may be elongated by adding a new sugar unit. Also a pre-elongated sugar chain prepared by adding a sugar unit to the trisaccharide may be introduced to the desired protein.

Alternatively, a protein sequence may be elongated when

standard peptide chemistry is applied to the asparagines residue in the asparagines-linked trisaccharide derivative. Also, a sugar chain may be elongated when standard carbohydrate chemistry is applied to mannose in the reducing terminal.

EXAMPLES

The present invention is explained in more detail by the following experiments, but not limited to them.

Materials used in the experiments are obtained from the commercial source described below.

SANSHO Co., Ltd. (Food Division)

Guargum MEYPROGAT 120S

KANTO CHEMICAL CO., INC

Zinc Powder

Copper (II) Sulfate Pentahydrate (crystalline powder)

Diammonium Cerium(IV) Nitrate

Wako Pure Chemical Industries, Ltd.

Sodium Acetate

Acetic Anhydride

Trifluoroacetic Acid

Sodium Azide

DBU, 1,8-Diazabicyclo[5, 4, 0]undec-7-ene

CCl_3CN, Trichloroacetonitrile

BF_3OEt_2, Boron Trifluoride Diethyl Ether Complex

Acetic Acid, For Organic Synthesis

Pyridine, For Organic Synthesis

Tetrahydrofuran, THF, For Organic Synthesis

CH_2Cl_2, Dichloromethane, For Organic Synthesis

Acetonitrile, For Organic Synthesis

Ethyl Acetate

Chloroform

Toluene

Anhydrous $MgSO_4$

Triethylamine

Tokyo Chemical Industry Co., Ltd.

30% HBr-AcOH, 30% Hydrogen Bromide in Acetic Acid

Nacalai Tesque, Inc.

Benzylamine

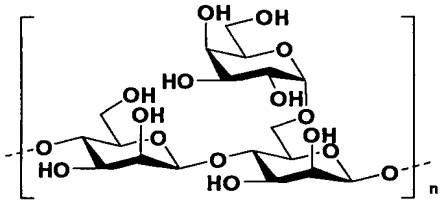
Japan Alcohol Trading CO., LTD

99% Ethanol

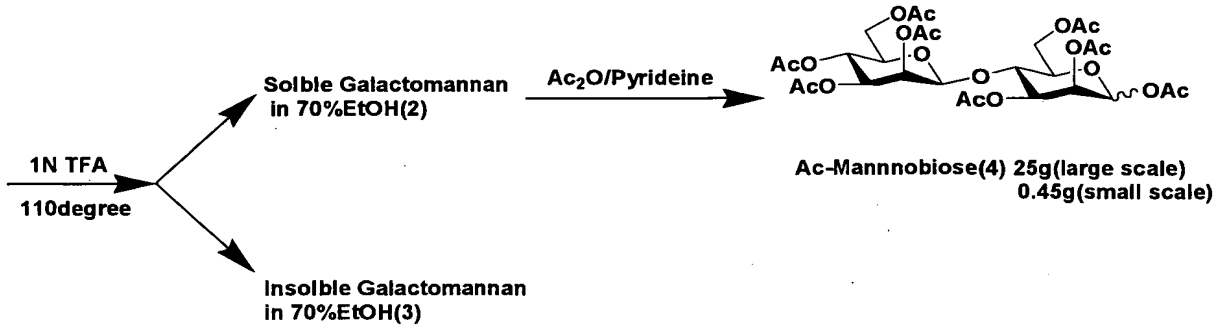
Example 1

1. Hydrolysis of Guar gum and isolation of Man β 1→4Man

Synthetic Scheme (1)



Guargum (1) 200g(large scale)
2.0g(small scale)



1.1. Hydrolysis of Guar Gum (a small scale)

(A) Galactomannan soluble in 70% EtOH is obtained by hydrolysis of

guar gum with TFA.

2.0g of guar gum (1) was dissolved in 16.6ml of 1N TFA and heated to 110°C in an oily bath with stirring for 90 min. The reaction mixture was poured into 40ml of 99% EtOH and placed at room temperature. The resulting white precipitation was removed by filtration using Buchner funnel and the filtrate was concentrated in vacuo. Toluene was added to the residue and azeotropically distilled several times to give 2.26g of galactomannan (2) soluble in 70% EtOH and 73mg of galactomannan (3) insoluble in 70% EtOH.

Analysis of galactomannan (2) soluble in 70% EtOH using MALDI-TOFMS showed that its polymerization degree was reduced to 1-8.

(B) Galactomannan soluble in 70% EtOH is acetylated to give O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-1,2,3,6-tetra-O-acetyl-α-and β-D-mannopyranoside (4).

2.26g of galactomannan (2) soluble in 70% EtOH obtained above was dissolved in 23ml of pyridine, 23ml of acetic anhydride was added in the solution with cooling in an ice bath and stirred at 10°C for 22 hours. To the reaction mixture was added ice water and extracted with chloroform, washed with water, aq. solution of $NaHCO_3$ and aq. solution of NaCl successively, and dried over anhydrous $MgSO_4$. After $MgSO_4$ was removed by Celite-filtration, the filtrate was concentrated in vacuo and the resulting residue was purified with silica-gel column chromatography (elution: toluene/ethyl acetate=2/1) to give 450mg of the desired product (4).

Sample; a mixture ofα:β = 2:1 ; [α]D-0.5 (c 0.012, chloroform); ^{1}H NMR δ ($CDCl_3$) 1.99~2.19 (all s, 24H, 8$COCH_3$), 3.64 (m, 1H, H-5'), 3.77 (m, 1/3H, H-5β),3.95~4.13 (m, 2+2/3H, H-5α, H-4β, H-4α and H-6'b), 4.23~4.37 (m, 3H, H-6bβ, H-6aα, H-6bα, H-6a' and H-6aβ), 4.72 (d,

1/3H, $J^{\beta}_{1',2'}$=1.1Hz, H-1β'), 4.75 (d, 2/3H, $J^{\alpha}_{1',2'}$=1.1 Hz, H-1α'), 5.04 (m, 1H, H-3'), 5.17~5.25 (m, 2H, H-4', H-2α and H-3β), 5.39~5.45 (m, 2H, H-2', H-2β and H-3α), 5.81 (d, 1/3H, $J^{\beta}_{1,2}$ = 1.1 Hz, H-1β), 6.03 (d, 2/3H, $J^{\alpha}_{1,2}$ = 2.0 Hz, H-1α).

Analysis calculated for $C_{28}H_{38}O_{19}$: C, 49.56; H, 5.64; Found: C, 49.34; H, 5.67.

HR-FAB MS[M+Na]$^+$Calculated for $C_{28}H_{38}O_{19}Na$:701.191; Found 709.190.

t.l.c; Rf = 0.30 (toluene/ethyl acetate = 1:1)

1.2. Hydrolysis of Guar Gum (a large scale)

(A)' Galactomannan soluble in 70% EtOH is obtained by hydrolysis of guar gum with TFA.

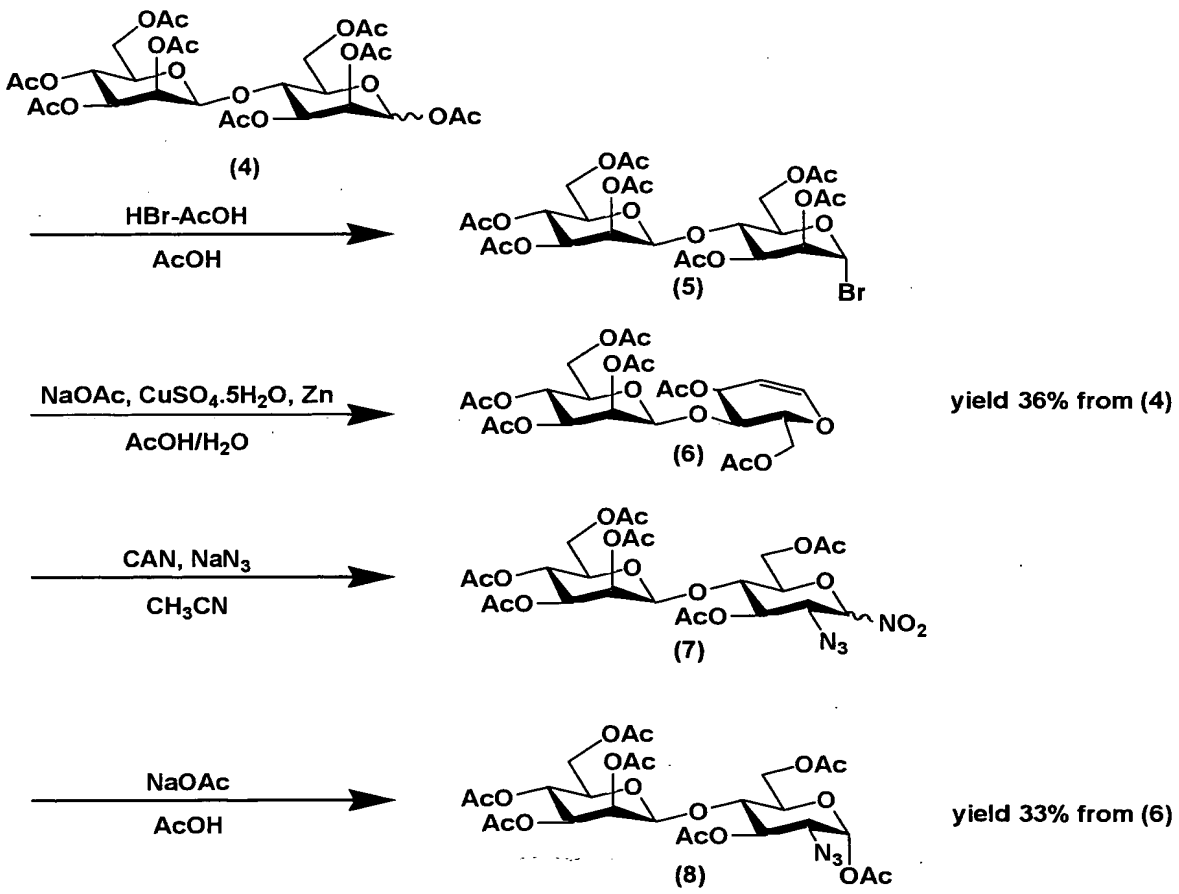
200g of guar gum (1) was dissolved in 1660ml of 1N TFA and heated to 110°C in an oily bath with mechanical stirring for 35 min. When the guar gum was suspended, the mixture was sonicated for 15min and mechanically stirred at 110°C for 80min. The reaction mixture was cooled with an ice bath, poured into 4 liter of 99% EtOH and placed at room temperature. The resulting white precipitation was removed by filtration using Buchner funnel and the filtrate was concentrated in vacuo. Toluene was added to the residue and azeotropically distilled several times to give 200.3g of galactomannan (2) soluble in 70% EtOH and 9.9g of galactomannan (3) insoluble in 70% EtOH. Analysis of galactomannan (2) soluble in 70% EtOH using MALDI-TOFMS showed that its polymerization degree was reduced to 1-8.

(B)' Galactomannan soluble in 70% EtOH is acetylated to give O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-1,2,3,6-tetra-O-acetyl-α-and β-D-mannopyranoside (4).

200.3g of galactomannan (2) soluble in 70% EtOH obtained above was dissolved in 2100ml of pyridine, 2100ml of acetic anhydride was added in the solution with cooling in an ice bath and stirred at 10°C for 22 hours. To the reaction mixture was added ice water and extracted with chloroform, washed with water, aq. solution of $NaHCO_3$ and aq. solution of NaCl successively, and dried over anhydrous $MgSO_4$. After $MgSO_4$ was removed by Celite-filtration, the filtrate was concentrated in vacuo and the resulting residue was partially purified with silica-gel column chromatography (elution: toluene/ethyl acetate=1/2), and then purified with the column chromatography of medium pressure (elution: toluene/ethyl acetate=2/1) to give 25.2g of the desired product (4).

2. Synthesis of O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-1,3,6-tri-O-acetyl-2-azide-2-deoxy-α-D-glucopyranoside (8)

Synthetic Scheme (2)



(C) Synthesis of O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-2,3,6-tri-O-acetyl-α-D-mannopyranosyl bromide (5)

2.20g of the compound (4) was dissolved in 19ml of acetic acid, and to the solution was added 4.6ml of 30% HBr-AcOH and the mixture was stirred at r.t. for 150 min in a dark place. After termination of the reaction was confirmed on t.l.c., ice water was added to the reaction mixture and the product was extracted with chloroform, washed with water, aq. solution of $NaHCO_3$ and aq. solution of NaCl successively, and dried over anhydrous $MgSO_4$. After Mg SO_4 was removed by celite-filtration, the filtrate was concentrated in vacuo to give 2.21g of the residual mixture containing the desired product (5).

t.l.c.; Rf = 0.35 (toluene/AcOEt = 1:1)

(D) Synthesis of O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-

3,6-di-O-acetyl-D-glycol (6)

Into a three-neck flask cooled in ice water bath, were added 3.8ml of acetic acid, 7.6ml of water, 2.06g of sodium acetate, 0.20g of cupper sulfate pentahydrates and 1.65g of zinc successively while being stirred with a mechanistic stirrer. Next, the reaction mixture containing the compound (5) was dissolved in 7.6ml of acetic acid, and it was added to the reaction mixture above cooled in an ice water bath and the mixture was stirred at r.t. for 4 hours in a dark place. After termination of the reaction was confirmed on t.l.c., zinc was removed from the reaction mixture by celite-filtration and ice water was added to the filtrate. The product was extracted with chloroform, washed with water, aq. solution of $NaHCO_3$ and aq. solution of NaCl successively, and dried over anhydrous $MgSO_4$. After $MgSO_4$ was removed by celite-filtration and the filtrate was concentrated in vacuo. The residue was purified with a flash silica gel chromatography (elution: toluene/ethyl acetate=2/1) to give 0.64g of the desired product (6).

Yield from the compound (4): 36%.

^{1}H NMR δ($CDCl_3$) .200, 2.05, 2.08, 2.10, 2.12 and 2.17(all s, 18H, 6$COCH_3$), 3.66(ddd, 1H, $J_{4',5'}$=9.8Hz, $J_{5',6a'}$ = 5.8Hz, $J_{5',6b'}$ =12.2Hz, H-5'), 4.05(dd, 1H, $J_{3,4}$=6.0Hz, $J_{4,5}$=8.1Hz, H-4), 4.12(dd, 1H, $J_{5',6b'}$=2.6Hz, $J_{6a',6b'}$=12.2Hz, H-6b'), 4.13-4.17(m, 1H, H-5), 4.23(dd, 1H, $J_{5,6b}$ =5.3Hz, $J_{6a,6b}$ =12.2Hz, H-6b), 4.30(dd, 1H, $J_{5',6a'}$=5.8Hz, $J_{6a',6b'}$ =12.2Hz, H-6a'), 4.42(dd, 1H, $J_{5,6a}$ =2.9Hz, $J_{6a,6b}$ =12.2Hz, H-6a), 4.79(dd,1H,$J_{1,2}$=6.1Hz, $J_{2,3}$ =3.1Hz, H-2), 4.86 (d, 1H, $J_{1',2'}$ =1.1Hz, H-1'), 5.05 (dd,1H, $J_{2',3'}$=3.4Hz, $J_{3',4'}$ =10.1Hz, H-3'), 5.22 (t, 1H, $J_{4',5'}$ =9.8Hz,H-4'), 5.45 (dd, 1H, $J_{1',2'}$ =1.1Hz $J_{2',3'}$ = 3.4Hz, H-2'), 5.51 (m, 1H, H-3), 6.40 (dd, 1H, $J_{1,2}$ =6.1 Hz, $J_{\text{duble bond cis}}$= 1.2Hz,H-1)

^{13}C NMR δ($CDCl_3$) 20.5-21.0(m, 6$COCH_3$), 61.8(C-6), 62.5(C-6'), 65.9(C-

4'), 68.5(C-3 and C-2'), 70.8(C-3'), 72.6(C-5'), 74.0(C-4), 74.4(C-5), 97.9(C-1'), 99.0(C-2), 145.6(C-1), 169.5-170.6(m, $6COCH_3$)

t.l.c.; Rf = 0.40 (toluene/ethyl acetate = 1:1)

(E) Synthesis of O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-3,6-di-O-acetyl-2-azide-2-deoxy α-and β-D-glucopyranosyl nitrate (7)

510mg of the compound (6) was dissolved in 5.4ml of anhydrous acetonitrile and stirred at -20°C. To the solution, 89mg of sodium azide (NaN_3) was added, and then 1.50g of cerium (IV) diammonium nitrate was added in four portions every 15 minutes. The reaction mixture was stirred under helium atmosphere at -20°C for 18 hours. After termination of the reaction was confirmed on t.l.c., ice water was added to the reaction mixture and the product was extracted with chloroform, washed with water, aq. solution of $NaHCO_3$ and aq. solution of NaCl successively, and dried over anhydrous $MgSO_4$. After $MgSO_4$ was removed by celite-filtration, the filtrate was concentrated in vacuo to give 460mg of the residue containing the desired product (7).

t.l.c.; Rf = 0.50 (toluene/AcOEt = 1:1)

(F) Synthesis of O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-1,3,6-tri-O-acetyl-2-azide-2-deoxy-α-D-glucopyranoside (8)

460mg of the residue containing the compound (7) was dissolved in 2.0ml of acetic acid, and to the solution was added 170mg of sodium acetate and stirred in an oil bath at 80°C for 75 minutes. After termination of the reaction was confirmed on t.l.c., ice water was added to the reaction mixture and the product was extracted with chloroform, washed with water, aq. solution of $NaHCO_3$ and aq. solution of NaCl successively, and dried over anhydrous $MgSO_4$. After $MgSO_4$ was removed by celite-filtration and the filtrate was concentrated in vacuo. The residue was purified with a flash silica gel chromatography (elution:

toluene/ethyl acetate=3/2) to give 360mg of the residue containing the desired product (8) and O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-1,3,6-tri-O-acetyl-2-azide-2-deoxy-α-D-mannopyranoside (9). The mixture was dissolved in a small amount of EtOH with heating, and then cooled in ice water to give a crystalline. Thus, 201mg of the desired product was obtained.

t.l.c.; Rf = 0.39 (toluene/AcOEt = 1:1)

Yield from the compound (6): 33%.

^{1}H NMR δ($CDCl_3$) 1.99, 2.05, 2.10, 2.12, 2.17 and 2.20 (all s, 21H, 7$COCH_3$), 3.51 (dd, 1H, $J_{1,2}$=3.8Hz $J_{2,3}$ = 10.5Hz, H-2), 3.61 (ddd, 1H, $J_{4',5'}$ =9.9Hz, $J_{5',6a'}$ =5.0Hz, $J_{5',6b'}$= 2.8Hz, H-5'), 3.83 (t, 1H, $J_{4,5}$ =10.2Hz, H-4), 3.99 (m, 1H, H-5), 4.12 (dd, 1H, $J_{5',6b'}$= 2.8Hz $J_{6a',6b'}$ = 12.3Hz, H-6b'), 4.24 (dd, 1H, $J_{5,6b}$= 3.7Hz $J_{6a,6b}$ = 12.5Hz, H-6b), 4.30(dd, 1H, $J_{5,6a}$=2.8Hz, $J_{6a,6b}$=12.5Hz, H-6a) 4.38(dd, 1H, $J_{5,6a}$=2.8Hz, $J_{6a,6b}$= 12.5Hz, H-6a), 4.66 (d, 1H, $J_{1',2'}$ = 0.6Hz, H-1'), 5.03 (dd, 1H, $J_{2',3'}$=3.2Hz, $J_{3',4'}$ = 9.9Hz, H-3'), 5.23 (t, 1H, $J_{4',5'}$ = 9.9Hz, H-4'), 5.42 (dd, 1H, $J_{1'2'}$=0.6Hz, $J_{2',3'}$ = 3.2Hz, H-2'), 5.43 (dd, 1H, $J_{2,3}$=10.5Hz, $J_{3,4}$ = 9.3Hz, H-3), 6.24 (d, 1H, $J_{1,2}$ = 3.8Hz, H-1)

^{13}C NMR δ($CDCl_3$) 20.5-20.9(m, 6$COCH_3$), 60.3(C-2), 61.9(C-6), 62.2(C-6'), 65.8(C-4'), 68.1(C-2'), 69.7(C-3), 70.4(C-5), 70.7(C-3'), 72.5(C-5'), 74.0(C-4), 89.9(C-1), 97.5(C-1'), 168.6-170.4(m, 6$COCH_3$)

Analysis calculated for $C_{26}H_{35}O_{17}$: C, 47.20; H, 5.33; N, 6.35; Found: C, 46.90; H, 5.32; N, 6.39.

HR-FAB MS$[M+H]^+$ Calculated for $C_{26}H_{36}N_3O_{17}$ 662.205, Found 662.202

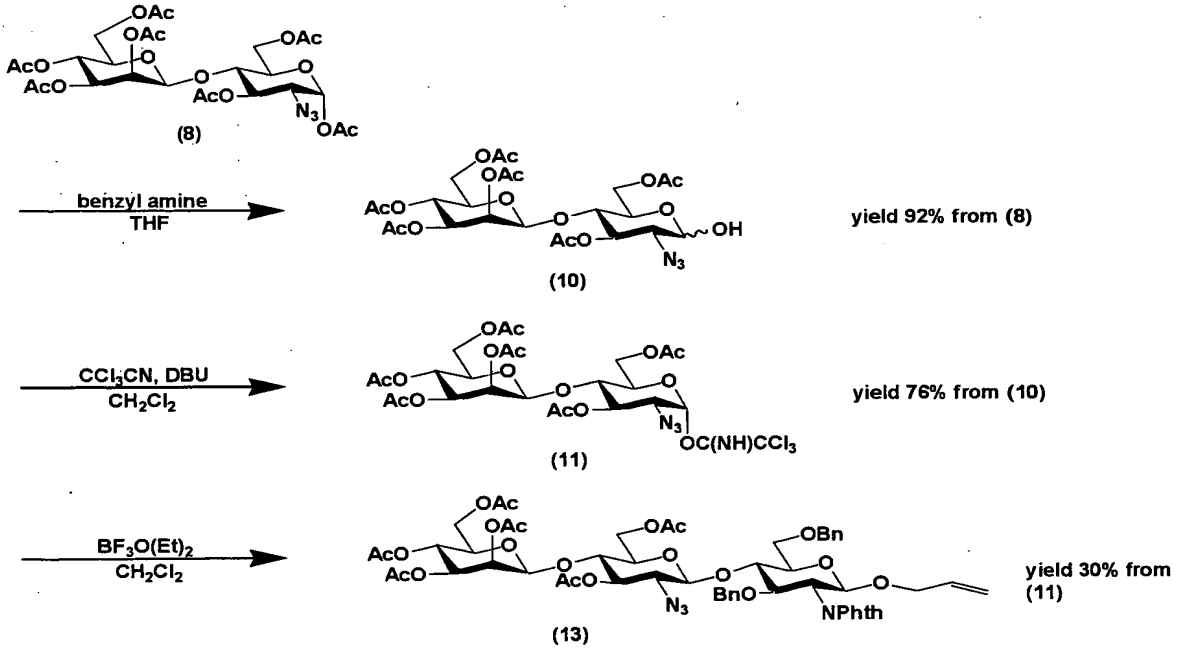
mp+183.5-184.0℃(from EtOH),

t.l.c.; Rf = 0.39 (toluene/ethyl acetate = 1:1)

3. Synthesis of allyl O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-O-(3,6-di-O-acetyl-2-azide-2-deoxy-β-D-

glucopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimide- β -D-glucopyranoside (13)

Synthetic scheme (3)



(G) synthesis of O-(2,3,4,6-tetra-O-acetyl- β -D-mannopyranosyl)-(1→4)-3,6-di-O-acetyl-2-azide-2-deoxy-D-glucopyranose (10)

300mg of the compound (8) was dissolved in 3.0ml of THF, and to the solution cooled in ice water was added 89μl of benzylamine and stirred at r.t. for 48 hours. After termination of the reaction was confirmed on t.l.c., ice water was added to the reaction mixture and the product was extracted with chloroform, washed with water, 1N HCl and aq. solution of NaCl successively, and dried over anhydrous $MgSO_4$. After $MgSO_4$ was removed by celite-filtration and the filtrate was concentrated in vacuo. The residue was purified with a flash silica gel chromatography (elution: toluene/ethyl acetate=3/2) to give 257mg of the desired product (10).

Yield from the compound (8): 92%.

HR-FAB MS$[M+H]^+$ Calculated for $C_{24}H_{34}N_3O_{16}$ 620.194; Found 620.192.

t.l.c; Rf = 0.26 (toluene/AcOEt = 1:1)

(H) Synthesis of O-(2,3,4,6-tetra-O-acetyl- β -D-mannopyranosyl)-(1→4)-3,6-di-O-acetyl-2-azide-2-deoxy- α -D-glucopyranosil trichloroacetoimidate (11)

85mg of the compound (10) was dissolved in CH_2Cl_2 (550μl) and CCl_3CN (275μl), and to the solution cooled in ice water was added 10.2μl of DBU and stirred at r.t. for 2 hours. After termination of the reaction was confirmed on t.l.c., the reaction mixture was concentrated in vacuo. The resulting residue was purified with a flash silica gel chromatography (elution: toluene/ethyl acetate=3/2) to give 80mg of the desired product (11).

Yield from the compound (10): 76%.

^{1}H NMR δ($CDCl_3$) 1.97, 2.02, 2.07, 2.08, 2.16 and 2.17(all s, 18H, $6COCH_3$), 3.56-3.60(m, 1H, H-5'), 3.60(dd, 1H, $J_{1,2}$=3.4Hz, $J_{2,3}$=10.5Hz, H-2), 3.88(t, 1H, $J_{4,5}$=9.8Hz, H-4), 4.09(dd, 1H, $J_{5',6b'}$=2.7Hz, $J_{6a',6b'}$=12.5Hz, H-6b'), 4.09-4.14(m, 1H, H-5), 4.21(dd, 1H, $J_{5,6b}$=3.9Hz, $J_{6a,6b}$=12.5Hz, H-6b), 4.33(dd, 1H, $J_{5,6a}$=2.2Hz, $J_{6a,6b}$=12.5Hz, H-6a), 4.33(dd, 1H, $J_{5',6a'}$=4.9Hz, $J_{6a',6b'}$=12.5Hz, H-6a'), 4.69(s, 1H, H-1'), 5.01(dd, 1H, $J_{2',3'}$=3.4Hz, $J_{3',4'}$=10.0Hz, H-3'), 5.20(t, 1H, $J_{4',5'}$=9.8Hz, H-4'), 5.38(d, 1H, $J_{2',3'}$=3.4Hz, H-2'), 5.51(dd, 1H, $J_{2,3}$=10.5Hz, $J_{3,4}$=9.5Hz, H-3), 6.41(d, 1H, $J_{1,2}$=3.4Hz, H-1), 8.79(s, 1H, NH)

^{13}C NMR δ($CDCl_3$) 20.5-20.7(m, $6COCH_3$), 60.8(C-2), 61.9(C-6), 62.3(C-6'), 65.8(C-4'), 68.2(C-2'), 69.3(C-3), 70.7(C-5 and C-3'), 72.6(C-5'), 74.1(C-4), 90.5($C(NH)CCl_3$), 94.1(C-1), 97.3(C-1'), 160.6($C(NH)CCl_3$), 169.5-170.4(m, $6COCH_3$)

t.l.c; Rf = 0.37 (toluene/ethyl acetate = 1:1)

(I) Synthesis of allyl O-(2,3,4,6-tetra-O-acetyl- β -D-mannopyranosyl)-(1→4)-O-(3,6-di-O-acetyl-2-azide-2-deoxy- β -D-glucopyranosyl)-(1→4)-

3,6-di-O-benzyl-2-deoxy-2-phthalimide-β-D-glucopyranoside (13)

47mg of the compound (11) and 45mg of allyl-O-3,6-O-di-benzyl-2-deoxy-2-phthalimide-β-D-glucopyranoside (12) were dissolved in 700μl of CH_2Cl_2, and to the solution was added 70mg of MS4A (Molecular Sieves) and stirred under nitrogen atmosphere at -20°C for 30 minutes.

Next, 2.3μl of BF_3OEt_2 was added and the mixture was stirred under nitrogen atmosphere at -20°C for 24 hours. After termination of the reaction was confirmed on t.l.c., the reaction mixture was neutralized by adding triethylamine (TEA), MS4A was removed by celite filtration and the filtrate was concentrated in vacuo. The resulting residue was partially purified with a flash silica gel chromatography (elution: toluene/ethyl acetate=5/2) to give 32mg of the residual mixture containing the desired compound (13) and allyl O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)-(1→4)-O-(3,6-di-O-acetyl-2-azide-2-deoxy-α-D-glucopyranosyl)-(1→4)-3,6-di-O-benzyl-2-deoxy-2-phthalimide-β-D-glucopyranoside (α:β=1:2). Furthermore, it was purified with HPLC (elution: hexane/ethanol=12/1) to give 21mg of the desired product (13).

Yield from the compound (11): 31%

^{1}H NMR δ($CDCl_3$) 1.90, 1.92, 1.96, 2.00, 2.07, 2.15(all s, 18H, $6COCH_3$), 3.08(m, 1H, H-5'), 3.27(dd, 1H, $J_{1',2'}$=8.1Hz, $J_{2',3'}$=10.2Hz, H-2'), 3.46(ddd, 1H, $J_{4'',5''}$=9.9Hz, $J_{5'',6a''}$=4.8Hz, $J_{5'',6b''}$=2.6Hz, H-5''), 3.51-3.56(m, 1H, H-5), 3.56(t, 1H, $J_{4',5'}$=9.8Hz, H-4'), 3.76(dd, 1H, $J_{5,6b}$=1.4Hz, $J_{6a,6b}$=10.9Hz, H-6b), 3.87(dd, 1H, $J_{5,6a}$=2.9Hz, $J_{6a,6b}$=10.9Hz, H-6a), 3.91(dd, 1H, J=6.3Hz, J=13.0Hz, CHH'CH=CH2), 3.98-4.20(m, 7H, H-6b'', H-6b', H-4, H-2, H-6a', H-3, CHH'CH=CH2), 4.27(dd, 1H, $J_{5'',6a''}$=4.8Hz, $J_{6a'',6b''}$=12.3Hz, H-6a''), 4.27 and 4.66(ABq, 2H, J=12.5Hz,

$PhCH_2$), 4.30(d, 1H, $J_{1',2'}$=8.1Hz, H-1'), 4.42 and 4.73(ABq, 2H, J=12.0Hz, $PhCH_2$), 4.45(s, 1H, H-1"), 4.76(dd, 1H, $J_{2',3'}$=3.4Hz, $J_{3',4'}$=9.9Hz, H-3'), 4.92(dd, 1H, $J_{2",3"}$=3.4Hz, $J_{3",4"}$=9.9Hz, H-3"), 4.93(dd, 1H, J=1.5Hz, J_{trans}=10.4Hz, $CH=CH_{trans}H$), 4.93(dd, 1H, J=1.5Hz, J_{cis}=17.2Hz, CH=CHHcis), 5.06(d, 1H, $J_{1,2}$=8.4Hz, H-1), 5.14(t, 1H, $J_{4",5"}$=9.9Hz, H-4"), 5.30(d, 1H, $J_{2",3"}$=3.4Hz, H-2"), 5.60(m, 1H, CH=CH2), 6.70-7.58(m, 14H, Ar-H)

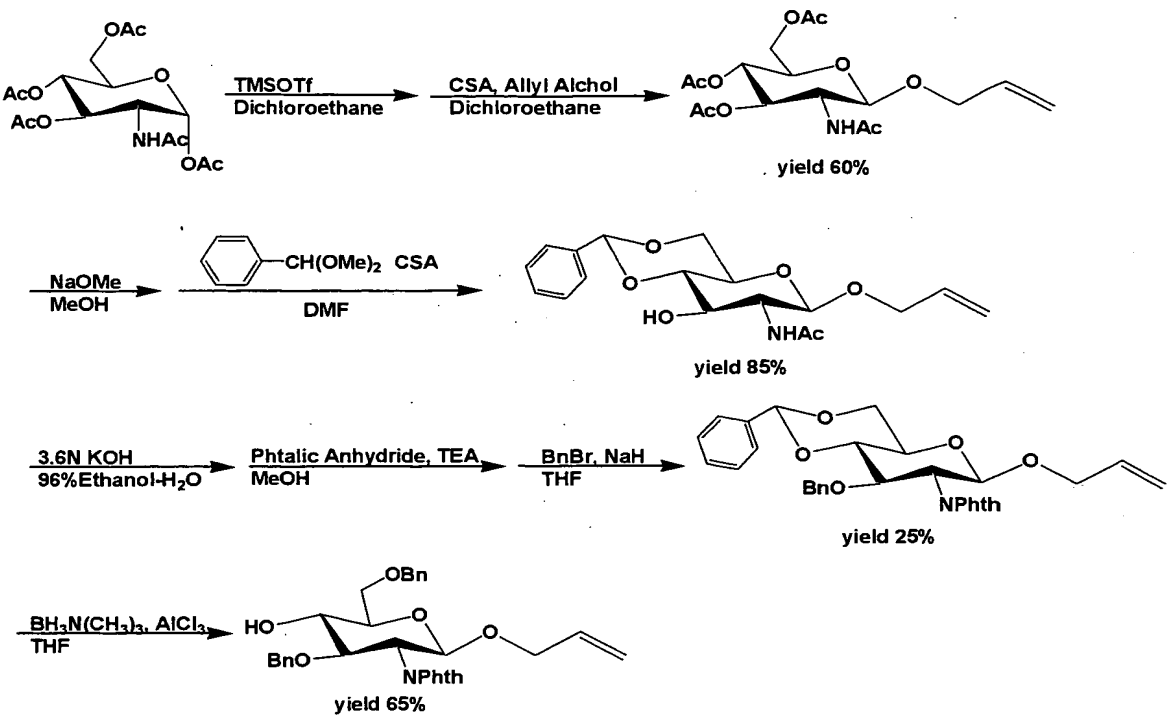
^{13}C NMR δ($CDCl_3$) 20.5-20.6(m, 6COCH3), 55.5(C-2), 62.2(C-6' and C-6"), 64.5(C-2'), 65.9(C-4"), 67.8(C-6), 68.1(C-2"), 69.7($CH_2CH=CH_2$), 70.7(C-3"), 71.9(C-3'), 72.0(C-5'), 72.5(C-5"), 73.5 and 74.3(2PhCH2), 74.6(C'-4 and C-5), 78.2(C-4), 97.3(C-1 and C-1"), 100.8(C-1'), 117.3($CH_2CH=CH_2$), 127.0-133.7(m, 18Ar-C), 137.9($CH_2CH=CH_2$), 169.6-170.4(m, 8C=O)

HR-FAB MS$[M+Na]^+$Calculated for $C_{55}H_{62}N_4O_{22}Na$, 1153.375, Found 1153.374

t.l.c; Rf = 0.53 (toluene/ethyl acetate = 1:1)

(J) (data of glycosyl acceptor) allyl-O-3,6-di-O-benzyl-2-deoxy-2-phthalimide-β-D-glucopyranoside (12)

Amino-protected glucopyranoside (12) was synthesized according to the synthetic scheme shown below.



^{1}H NMR δ($CDCl_3$) 3.63(m, 1H, H-5), 3.76-3.85(m, 3H, H-4, H-6a and H-6b), 3.97(dd, 1H, J=13.1Hz, J=6.1Hz, CHH'CH=CH_2), 4.15-4.26(m, 3H, H-2, H-3 and CHH'CH=CH_2), 4.52 and 4.73(ABq, 2H, J=12.2Hz, PhCH2), 4.58 and 4.64(ABq, 2H, J=11.9Hz, PhCH2), 4.99(dd, 1H, J=1.3Hz, J_{trans}=10.3Hz, CH=CHcisH_{trans}), 5.07(dd, 1H, J=1.3Hz, J_{cis}=17.2Hz, CH=CH_{cis}Htrans), 5.17(d, 1H, $J_{1,2}$=8.1Hz, H-1), 5.61-5.70(m, 1H, CH=CH_2), 6.93-7.67(m, 14H, Ar-H)

^{13}C NMR δ($CDCl_3$) 55.3(C-2), 69.7(C-C=C), 70.7(C-6), 73.5(C-5), 73.8 and 74.3(Ph-C), 74.5(C-4), 78.7(C-3), 97.4(C-1), 117.3(C-C=C), 127.4-128.5(m, Ar-C), 133.6(C-C=C), 137.6 and 138.2(C=O)

HR-FAB MS$[M+H]^+$Calculated for $C_{31}H_{32}NO_7$ 530.218, Found 530.215

t.l.c; Rf = 0.72 (toluene/ethyl acetate = 1:1)

INDUSTRIAL APPLICABILITY

Glycosyltransferase and an additive sugar unit are usually utilized in the automatic synthesizer of sugar chains since

glycosyltransferase is convenient when a sugar chain is extended by adding a new saccharide. However, no glycosyltransferase is found to prepare the trisaccharide moiety (Manβ1→4GlcNβ1→4GlcN) of the reducing terminal in the core sugar chain structure of asparagine-linked glycoprotein and the chemical synthesis is the sole method for preparing it.

The present invention provides a convenient method for preparing the trisaccharide moiety of the reducing terminal in the core sugar chain utilizing galactomannan, guar gum and/or mannan derivatives, which are natural polysaccharides easily available at a reasonable cost.